Searching for Prostate Cancer Stem Cells: Markers and Methods

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Abstract

The cancer stem cell hypothesis postulates that a single stem-like cancer cell is able to produce all cancer cell types found in a tumour. These cells are also thought to be the causative agents of relapse following therapy. In order to confirm the importance of cancer stem cells in tumour formation and patient prognosis, their role in prostate cancer must be comprehensively studied. This review describes current methods and markers for isolating and characterizing prostate cancer stem cells – including assays for self-renewal, multipotency and resistance to therapy. In particular the advantages and limitations of these approaches are analysed. The review will also examine novel methods for studying the lineage of cancer stem cells in vivo using transgenic mouse models. These lineage tracing approaches have significant advantages and, if a number of challenges can be addressed, offer great potential for understanding the significance of cancer stem cells in human prostate cancer.

Introduction

The cancer stem cell (CSC) hypothesis postulates that tumour masses may arise from a single cancer cell with stem-like characteristics. These CSCs are thought capable of self-renewal and differentiation to regenerate the tumour mass and all tumour cell types found within. Such cancer cells were first identified in leukemia in the 1990s (1,2) followed by discoveries in breast cancer (3), and subsequently in other solid tumours (4,5) including prostate cancer (6). CSCs appear to be rare within tumours as only a small proportion of all prostate cancer cells are able to reliably form large clones *in vitro* (6) and xenograft prostate tumours *in vivo* (7,8). Another feature attributable to cancer stem cells is their resistance to conventional treatment regimens, particularly chemotherapy (9) and the continued survival and self-renewal of CSCs is potentially an enticing explanation for relapse, metastasis and therapy failure in prostate cancer. A concerted effort is underway to isolate, characterise and target this critical cell population in order to produce more effective therapies.

In addition to the excitement surrounding CSCs there is also a great deal of controversy concerning their study. The putative CSC subpopulations isolated from prostate cancer cell lines and patient tumours vary considerably in their expression patterns and phenotypes. This heterogeneity complicates the identification of distinct cancer stem cell markers and impedes efforts to target the cells therapeutically. The hierarchy of cell fate in the normal prostate is also incompletely understood. For instance, the origin and ontogeny of normal adult prostate stem cells remains controversial (10), with some suggesting that there is a common progenitor of basal and luminal cells (11,12); that basal cells differentiate to produce luminal epithelium (12); or that basal and luminal

cells have separate progenitors to maintain their lineages (13). The origins of prostate cancer are also poorly understood, and there is evidence that prostate cancer may arise from both basal cells (14) and luminal cells (15) in murine models and in humans. Likewise, it is unclear whether cancer stem cells are transformed stem cells; or differentiated cancer cells that reactivate stem cell-associated self-renewal programs (10). Therefore, methods to probe both stem cell phenotypes and cancer cell fate are crucial to identify and characterise prostate cancer stem cells.

This review will focus on the markers and methods used to characterise cancer stem cells in the prostate (summarized in Figure 1). Methods to measure self-renewal, multipotency and resistance to therapy will be covered and the advantages and limitations of these methods examined. We will also consider recent novel approaches using in vivo lineage tracing that are beginning to be applied to the study of prostate cancer stem cells. These offer significant advantages over traditional methods, but there are still challenges that need to be overcome in the search for the ideal model to study prostate CSCs.

Biomarkers for Prostate Cancer Stem Cells

In this context, biomarkers are RNAs or proteins whose expression levels - either alone or in combination with other proteins – indicate the presence of cancer stem cells. Although there are many potential CSC markers, this review will cover some of the frequently used markers. Cell surface biomarkers are especially versatile because they can often be used to purify and analyse cancer stem cell populations by fluorescence-activated cell sorting (FACS) or magnetic-activated cell sorting (MACS). Expression of markers can also be analysed by immunohistochemistry and reverse-transcriptase

polymerase chain reaction (RT-PCR), often in combination with the above cell sorting methods.

Cell Surface Markers

Cell surface markers may be used to isolate cancer stem cells from a cell line or dissociated primary tumour. CD44 is a hyaluronan-binding cell-surface glycoprotein that is often used to purify cancer stem cells by FACS. CD44 has recently been the basis for the isolation of putative cancer stem cells in many tissues, including the prostate. When characterizing prostate cancer stem cells, CD44 is often complemented by a variety of other markers (Table 1).

CD44 and other cancer stem cell markers also help to verify that isolated populations are likely to be cancer stem cells. CD44 was found to be highly expressed in tumourspheres derived from four different prostate cancer cell lines (16). $CD44^+/CD24^-$ purified DU145 (17) and LNCaP (8) prostate cancer cell lines form prostatospheres (see self-renewal) with the potential to differentiate. Stem-like $PSA^{-/low}$ cells sorted for the antigenic profile $ALDH^+/CD44^+/ \alpha_2\beta_1^+$ had higher tumorigenicity than $ALDH^-/CD44^-/ \alpha_2\beta_1^-$ cells and PSA^- cells, with ten cells being sufficient to induce a xenograft tumour in NOD/SCID mice (18). Similarly, tumorigenic prostate cancer stem cells, comprising 0.1% of the total cells, have been isolated from primary human prostate tumours using the antigenic profile $CD44^+/\alpha_2\beta_1^{high}/CD133^+$ (6). Immunofluorescent staining may be employed to investigate the expression of markers such as CD44, CD24 and $\alpha_2\beta_1$ integrin for confirmation of stem-like phenotypes in tumourspheres, such as those generated from the DU145 cell line (19). Tumoursphere cells in this case were

$CD44^+/CD24^+/\ \alpha_2\beta_1^+$, in contrast to the $CD44^+/CD24^-$ profile mentioned previously. This suggests that there may be more than one group of stem-like cells to be found in cancer cell populations, and a single antigen - or panel of antigens - may be insufficient for isolating all cancer stem-like cells of interest. This has recently become apparent in an in vivo model of prostate cancer, where only $CD133^-$ basal-enriched human prostate epithelial cells were vulnerable to transformation, despite its use as a marker for CSCs (14). Similarly in head and neck squamous cell carcinoma, $CD44^-$ cells had CSC-like properties despite its use as a marker for CSCs (20). However, it is clear that CD44 and other cancer stem cell markers may be more useful when combined with other markers of interest.

Markers of Resistance

The ABC transporter ABCG2 can be used to identify populations of putative stem cells in many tissues, and is thought to be the molecular basis for the Hoechst side population, which is commonly used to select for chemoresistant cancer stem-like cells from many tumour types (21,22) (see Resistance to Therapy). ABCG2 may be involved in resistance to therapy, both from chemotherapy (22) and from androgen deprivation (23). However, as with many putative CSC markers the use of ABCG2 remains controversial. $ABCG2^-$ and $ABCG2^+$ prostate cancer cell line subpopulations can interconvert and are similarly tumorigenic, which makes it difficult to isolate distinct populations in vitro (24). Patrawala and colleagues propose that the $ABCG2^+$ population actually marks highly proliferative progenitor cells, while the $ABCG2^-$ cells represent cancer stem cells. Given this information, ABCG2 alone may not be the ideal marker for isolating cancer stem cells directly, but it is useful for identifying cells with probable therapeutic resistance phenotypes. Recently, ABCG2 has been used in a panel of markers

($CD133^+/CD44^+/ABCG2^+/CD24^-$) to isolate cancer stem cells in prostate cancer explants and prostate cancer tissue (25), suggesting that its use as a marker may be improved when used in tandem with other markers.

Markers of Pluripotency

Many embryonic stem (ES) cell pluripotency factors – such as Nanog, Oct4 and Sox2 - are known to be expressed in both adult stem cells and CSCs, and an embryonic stem cell gene expression signature was found to identify poorly differentiated tumours from several types of human cancer (26). Such factors may be useful for the identification of stem-like cells in prostate cancer, as they underpin important cancer stem cell phenotypes such as self-renewal. DU145 prostate tumoursphere cells express the ES pluripotency factors Nanog, Oct4 and Sox2 (19), as do immortalized prostate cancer epithelial cell cultures (27). Expression of Nanog and Oct4 is also enriched in primary tumours of gleason grade 5, an indicator of poor prognosis (28). In xenograft prostate tumours, rare $CD44^+$ stem-like cells have also been found to express Nanog (29). This evidence suggests that the presence of stem cell markers is a common feature of poor-prognosis tumours. However, Sox2 expression was recently found to be regulated by signaling through the Androgen Receptor (30), but was not associated with a CSC population. In addition both Oct4 (31) and Nanog (32,33) have multiple pseudogenes and splice variants which could have differing importance to prostate cancer stem cells and complicate analysis. Therefore, caution should be used when treating expression of these markers in isolation as evidence of cancer stem cells.

Biomarkers of Prostate Cell Lineage

Markers that identify specific cell types of the prostate are useful to characterise candidate prostate cancer stem cells. This is important as the normal prostate cell type

that prostate cancer stem cells most resemble remains controversial. For example, populations of castration resistant stem-like cells persist in cell lines *in vitro* (23,27) and in prostate cancer xenografts *in vivo* (29). Germann et al. have suggested that the CSCs found in the xenograft model are of luminal or possibly neuroendocrine cell origin, detecting elevated expression of the luminal marker Cytokeratin 18 and neuroendocrine cell marker Chromogranin A following castration (29). Conversely, another group observed upregulation of basal-specific cytokeratin 5 in DuCaP cell lines under androgen deprivation, but could not detect expression of the neuroendocrine marker Nestin (23). However, when comparing expression profiles of castration resistant and non-resistant human prostate tumours by microarray, the group found that both basal (Cytokeratin-5) and neuroendocrine (Nestin) markers were upregulated along with the putative cancer stem cell markers CD44 and c-Kit. Immortalised prostate cancer epithelial cells generated by Gu *et al.* (27) also express Nestin along with embryonic stem cell markers, consistent with a neuroendocrine-like CSC phenotype. The variability of expression patterns in multiple models of prostate cancer suggests that cancer stem cells may be more than one distinct subpopulation. It could also be a reflection of the phenotypic plasticity of cancer stem cells that has been recently indicated *in vitro* and *in vivo* (24,34,35). Regardless, determining the cell type of origin – and the heterogeneity thereof - is important in order to further establish the identity of prostate cancer stem cells.

Overall, biomarkers are useful for probing all aspects of cancer stem cells but given the apparent heterogeneous nature of cancer stem cells, no single marker is ideal and combinations of markers should be analysed in tandem for effective phenotypic

characterization. In addition these markers should be used in combination with functional assays which are considered below.

Self-Renewal

A defining property of both stem cells and cancer stem cells is their self-renewal capacity. There are two main types of in vitro assays used to probe self-renewal in isolated cell populations: clonogenicity assays and sphere formation assays. Both assays involve the culture of isolated cells in conditions that preferentially maintain stem cells, thus selecting for those cancer cells with more stem-like properties *in vitro*. They may also be considered proliferation assays, as self-renewal and proliferation are not distinct in this context. As most cancer cells are expected to have some self-renewal capacity, these assays are comparative in nature, and rely on the isolated CSCs being significantly different from a control population of cells for a tested phenotype. *In vitro* assays are not standalone tests for cancer stem cells and should be used in conjunction with other functional assays and marker analyses.

The clonogenicity assay involves seeding a single cell or a small number of cells onto culture plates, and monitoring colony formation after a defined time period. This assay is based on the assumption that small titres of cells will only form large colonies if they have the ability to self-renew (36). The number of colonies present is then treated as a correlate of self-renewal capacity. This assay can be done serially with a single cultured cell, making it possible to further demonstrate a clone's self-renewal capacity over multiple passages *in vitro* (6). There are some disadvantages with this assay; one being that an arbitrary threshold must be assigned to determine which colonies are too small

to count. More significantly the assay may not provide a complete representation of self-renewal capacity *in vivo*.

Another important tool is the sphere formation assay. The method was originally used to study adult neural stem cells *in vitro* (37) and has now been applied to isolate CSCs. Low adherence culture in defined serum-free medium produces clonal multicellular spheroid aggregates called tumourspheres (prostatospheres in prostate cancer), which are enriched for cells displaying cancer stem cell phenotypes. Anchorage-independent growth is a malignant phenotype in itself, suggesting that isolated CSCs are selected by their innately malignant phenotypes. This method is now widely-used for the enrichment of cancer stem cells from many types of cancer, including prostate cancer. The assay is versatile, and may be used in prostate cancer cell lines (16,38,39) and explanted primary prostate tumour tissue (25,39,40), or to further characterize cells isolated by other methods (41-43). Prostatospheres form at low efficiency, originating from a small fraction of the tumour cell population (40) and can be serially cultured (40,44-46), allowing for additional probing of self-renewal capacity and further enrichment of CSCs. Prostatosphere cells tend to possess CSC-associated phenotypes, such as self-renewal in long-term culture and expression of putative cancer stem cell markers such as CD44 and integrin $\alpha_2\beta_1$ (19,47). However, sphere formation may not always select for cancer stem cells *in vitro*. $\alpha_2\beta_1^+$ prostate cancer stem cells isolated from prostate xenograft tumours had high clonogenicity and sphere-forming capacity, but did not have the high tumorigenicity in xenografts expected of cancer stem cells (48). Similarly, when Matilainen *et al.* (49) attempted to isolate breast cancer stem cells using this method, the result was an unexpected reduction in CSC-associated gene expression and a loss of metastatic phenotypes. Prostatospheres may not consist purely

of CSCs, but contain some differentiated and dying cells (17) - and there are concerns that sphere generation is not being sufficiently validated – for example, to ensure that sphere formation is a result of cell proliferation and not cell aggregation (50). These conclusions highlight the need to verify all findings *in vivo* before any conclusions are made.

A well-established test for *in vivo* self-renewal comes from xenograft transplantation assays. Low titres of CSCs – isolated from cell lines (7) or from dissociated primary (7) and xenograft (40) prostate tumours are injected into immunodeficient mice. Cells that reliably form primary tumours (7,38,40,51) or metastases (7) at this limiting dilution are proposed to have a cancer stem cell phenotype. To provide more evidence for the CSC identity of the cells, xenograft tumour formation assays can be done serially using purified dissociated cells from xenograft tumours (51). The ability to serially generate tumours at limiting dilution is an indicator of self-renewal *in vivo*. Large tumour size and high tumour-forming capacity are indicators of an aggressive subpopulation of cells *in vivo*. Although this does not fully represent tumour formation in situ, it is a versatile and commonly used model for tumorigenesis and self-renewal *in vivo*. In fact this approach was perhaps the gold standard model for studying CSCs, until the advent of *in vivo* lineage tracing approaches, which are considered below.

Multipotency

Another defining characteristic of cancer and normal stem cells is their ability to differentiate. Differentiation can be induced *in vitro* by culturing cells in Matrigel™ (BD Biosciences), and multipotent prostate cancer cells may produce glandular structures that recapitulate prostate cancer architecture *in vivo* (27). Alternatively, CSCs may

differentiate in standard mammalian cell culture with added serum (6,8), which can be verified by the loss of expression of stem cell markers, and increased expression of differentiated cell markers.

Quantitative matrigel assays have been developed specifically for the study of prostate stem cell differentiation *in vivo* (52). Matrigel induces differentiation, causing putative prostate stem cells to form glandular structures. The group injected a single cell suspension of digested murine prostate in matrigel into the flank of BALB/c mice in order to assay for prostate reconstitution *in vivo*. Different proportions of EGFP-labeled transgenic mouse prostate cells and unlabeled mouse prostate cells were used to trace their respective contribution to reforming individual prostate ducts. No mosaicism was observed in the ducts, allowing them to propose that single prostate stem/progenitor cells are able to reconstitute entire prostate ducts, thus demonstrating the self-renewal and differentiation potential of the cells. In a similar tissue recombination assay, prostate cancer cells can be recombined with inductive rat urogenital mesenchyme and grafted under the renal capsule of immunodeficient mice. Gland-like structures form that recapitulate the structures and cell types found in the original prostate (53) or prostate tumour (27), thus demonstrating a stem-like capacity for differentiation. The renal graft model can also be used to assay for malignant transformation capacity, by recombining selected prostate epithelial cells with cancer-associated fibroblasts (14). The main advantage of these xenograft models is that the supporting stroma can also be altered to suit experimental needs. These approaches are beginning to elucidate the cellular origin of prostate cancer and the role that stromal interactions play in it.

Resistance to Therapy

The segregation of cancer stem cells from a cell population may be achieved by selecting for their resistance to standard cancer treatments. This is based on the observation that cancer stem cell populations tend to survive exposure to chemotherapeutic agents in vitro. Similarly, cancer stem cells have been proposed to be resistant to radiation and androgen withdrawal. If a resistant cancer stem cell population exists within prostate cancer then it could contribute to relapse from multiple treatments and is thus an enticing therapeutic target worthy of further analysis. Where possible, all aspects of therapeutic resistance must be characterized.

Prostate CSCs and Castration Resistance

Androgen deprivation is commonly used to treat metastatic prostate cancer, under which the majority of cancer cells will die, producing a remission. However, castration resistance inevitably follows in the period of months to years, resulting in relapse and metastasis. In an androgen-sensitive human xenograft model, Germann et al. have recently identified subpopulations of cancer cells that repopulate the tumour following a cycle of androgen deprivation and replacement (29), implying a potential role for cancer stem cells in the development of castration-resistant prostate cancer. Miki et al. have shown that inducing differentiation in culture can cause stem-like AR^- prostatosphere cells to become AR^+ (39), suggesting that lack of AR expression may be an important cancer stem cell phenotype. Therefore, testing for 'castration resistance' and AR expression are important characterization steps for any prostate cancer stem cell population.

Prostate CSCs and Chemoresistance

One method of isolating cells that are potentially chemoresistant and stem-like is the well-established Hoechst Side Population sorting protocol. In this method, live cells are stained with the dye Hoechst 33342. Cells stained with this dye fluoresce at blue and red wavelengths that can be detected by Flow Cytometry. In contrast, stem-like cells efflux this dye and fluoresce poorly in channels, allowing for their separation from the rest of the population by cell sorting (22), and further characterization. The ability to efflux cytotoxic Hoechst dye is linked to the expression of ABC transporters such as ABCG2 (21), which may also be involved in the efflux of chemotherapeutic drugs (22). Because of this, the method is able to identify chemoresistant stem-like cancer cells without prior knowledge of CSC expression profiles in the tumour type (54). One disadvantage of this approach is that the Hoechst dye is cytotoxic, so cells may not be amenable for further analysis.

Enzymes of the Aldehyde Dehydrogenase (ALDH) group are associated with poor prognosis in a number of cancers (55). Some aldehyde dehydrogenases catalyse the biosynthesis of retinoic acid, which is an important molecule involved in differentiation (55). They also participate in aldehyde and alcohol metabolism. Thus, ALDH enzymes may be functionally involved in self-renewal and resistance to alkylating agents such as cyclophosphamide (56). In the presence of the dye ALDEFLUOR® (STEMCELL Technologies), stem-like cells with high total ALDH activity will fluoresce green and can be sorted by flow cytometry. This method successfully isolates cancer stem cells from several human cancer types (54,55,57,58) including prostate cancer cell lines (36,44). ALDH1A1 and other stem cell markers are upregulated in castration resistant prostate cancer compared to noncastrated metastatic disease (23), suggesting a role for

ALDH1A1 in the response to androgen deprivation. Indeed, high ALDH1A1 expression in prostate cancer specimens has been associated with cancer stem-like phenotypes, higher gleason scores and stages, as well as a poor prognosis (51).

Prostate CSCs and Radiation Resistance

Some isolated cancer stem cells show resistance to ionizing radiation treatment. Cancer stem cells have been found to be radiation resistant in breast cancer cell lines (59,60) and glioblastoma (61). Currently this phenotype has not been widely characterized in prostate cancer stem cells. In the two prostate cancer cell lines that have been investigated, the radiation responses are variable (38), with LNCaP CSCs showing increased survival compared to total cells, and DU145 CSCs showing no difference. Although this work suggests a role for prostate CSCs in long term recovery from radiation, the mechanisms for this resistance are unknown. Therefore, more work is required to determine whether radiation resistance is a distinguishing characteristic of prostate cancer stem cells.

Novel Approaches *In Vivo*

Lineage tracing has recently been used to explore the nature of cancer stem cells *in vivo.* Lineage tracing is a crucial method for demonstrating the ability of a cell to give rise to progeny of different cell types and, when combined with mouse models of cancer, provides a powerful tool to study CSCs in a representative model of carcinogenesis.

Transgenic mouse models have the benefit of being more representive of carcinogenesis, as transformed cells can be investigated *in situ* – as opposed to xenograft models, where the tumour must be studied in a foreign tissue

microenvironment that has been disrupted. A comprehensive lineage tracing study by Wang *et al*. (12) employed lineage tracing to study stem cells in the murine prostate and prostate cancer. These mice expressed a tamoxifen-inducible Cre-recombinase under the control of a CK5 promoter. Upon administration of low doses of tamoxifen, recombination occurs – causing small numbers of basal cells to express YFP under the control of a CK5 promoter. This allows cell fate to be analysed at a single cell resolution. Using this method, they showed that some bipotential basal cells are capable of giving rise to luminal cells during repeated cycles of prostate regression and regeneration, as well as during normal prostate homeostasis. Using $Pten^{flox/flox}$ mice in this lineage tracing system, they were able to induce basal and luminal-derived prostate cancers – a finding that has been previously observed in similar lineage tracing studies of the murine prostate (13). Although these studies do not elucidate the exact lineage relationships underlying prostate cancer, they show that basal and luminal cells can act as targets of cellular transformation. Importantly, their results suggest that the cell type of origin may influence the latency and aggressiveness of the tumour - and potentially impact on clinical outcome of prostate cancer patients. Furthermore, they demonstrated that the progenitor cells were much more abundant in prostatospheres and renal grafts grown in vitro than in the transgenic prostate *in situ*. These findings exemplify the importance of high-quality *in vivo* models for the study of prostate cancer stem cells.

Lineage tracing can be further refined to resolve the changes of fate that occur throughout cancer development. Schepers et al traced $Lgr5^{+}$ intestinal adenoma stem cells in Apc-null mice (62), using a tamoxifen-inducible multicolour Cre reporter system that allowed them to follow the clonal development of each adenoma from single adenoma stem cells. Recombination was then induced a second time to follow the

dynamics of adenoma stem cell proliferation, dubbed lineage re-tracing. The re-tracing revealed that intestinal adenoma stem cells are associated with adenoma Paneth cells, as in the normal intestinal crypt, along with assumed transit-amplifying cells. Re-tracing thus allows for the observation of cancer stem cells in their resident niches, and might also enable the study of phenotypic plasticity in cancer stem cells *in vivo*. This technique has yet to be utilized in the study of prostate cancer.

Sophisticated lineage tracing analyses such as these allow for the study of cancer cell population dynamics to be studied at the level of single cancer stem cells, allowing the existence of cancer stem cells to be demonstrated in well-characterised *in vivo* models of a cancer. The approaches also have the advantage of interfering minimally with tumour development, eliminating potential artifacts caused by growing tumours in heterotopic positions or *in vitro*.

Hopefully, lineage tracing *in vivo* will become the gold standard for demonstrating the existence of cancer stem cells and validating cancer stem cell markers in the prostate. Encouragingly, transgenic mouse models have been successfully employed to trace the lineages of both luminal (63) and basal cells in the murine prostate and prostate cancer (12). Lineage tracing studies in the prostate are gradually discerning the cells of origin in prostate cancer and the nature of stem cells in the prostate. However, there are currently limitations to this approach. Mice do not spontaneously develop benign hyperplasia or prostate cancer and require transforming agents or genetic manipulation (such as deletion of Pten) to stimulate the tumorigenesis (64). There are also some discrepancies between human and transgenic murine prostate cancers. For example, murine models of prostate cancer are very unlikely to undergo metastasis to bone,

which is a common feature of the human disease (65). Anatomical dissimilarity between murine and human prostates is an additional obstacle - it has been previously shown that transgenic murine models of prostate cancer can vary in phenotype between prostate lobes (13). Nevertheless, transgenic mouse models are superior to xenografts for modeling the early stages of prostate cancer and are highly likely to facilitate significant future advances.

The ideal method for identifying cancer stem cells would allow for the examination of cancer stem cells in real time as the tumour develops. Non-invasive imaging methods allow for live tumour imaging and thus provide a wealth of data that would not be available through conventional marker analyses. Fluorescent imaging of the putative CSC marker CD133 has been achieved in subcutaneous xenograft tumours (66), using intravenous administration of fluorescently labeled anti-CD133 antibodies. When implanted subcutaneously, bioluminescent imaging of Balb/c mice has also successfully detected single luciferase-expressing cancer cells (67). There is the potential to adapt this for imaging of cancer stem cells, where detecting small numbers of cells is crucial. Imaging of deeper tissues is complicated by light scattering, which may limit its usefulness for live imaging of prostate cancer stem cells. However, the group were able to detect micrometastases, which demonstrates the ability of the technique to image at least some deep tissues. If this could be improved then the potential to combine single cell *in vivo* lineage analysis *and* live imaging offers great potential for future analysis of CSCs.

Conclusion

Cancer stem cells in the prostate are still poorly understood despite concerted efforts to identify and characterize them. There are established prostate cancer stem cell expression profiles, but heterogeneity and phenotypic plasticity hinder the use of a defined panel of markers in this manner. There are also well established in vitro and in vivo xenograft methods that can be extremely useful in the study cancer stem cells. However, these are not without limitations and it is perhaps the advent of *in vivo* lineage tracing methods which provides the most exciting opportunity to characterise prostate cancer stem cells in ever more innovative ways.

Conflict of Interest

No conflicts of interest have been declared.

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Figures

Figure 1.

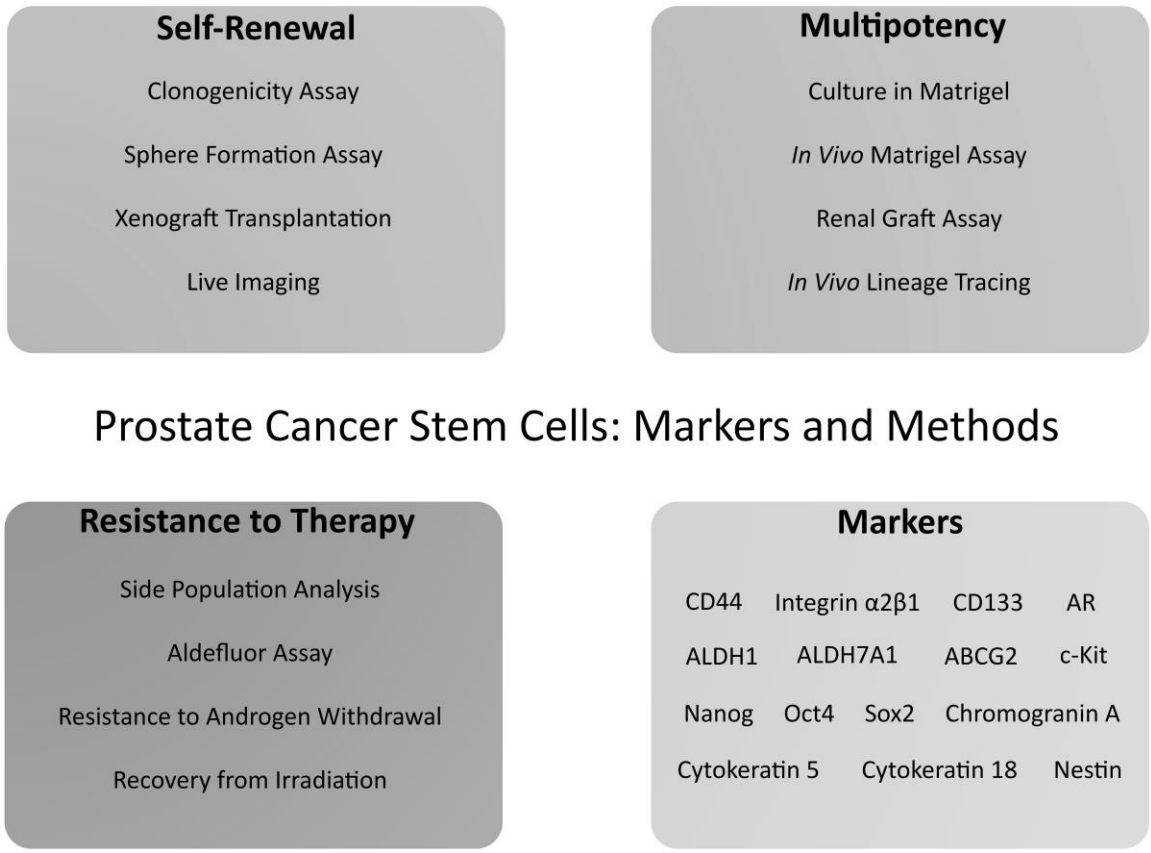

Table 1

Expression Profile	Cells/Tissues Studied	Evidence for Cancer Stem Cell Identity	Source
$CD44^+/CD24^-$	Prostate Cancer Cell Lines, Prostate Cancer Xenografts	Anchorage-independent growth; Increased clonogenicity; Prostatosphere Formation; Expression of stem cell markers; Tumour formation in mice at low titres.	(8)
$CD44^+/CD133^+/\ \alpha_2\beta_1^{high}$	Explanted Primary Human Prostate Tumour	Anchorage-independent growth; Increased clonogenicity; Cells differentiate upon culture with serum.	(6)
$CD44^+/CD133^+/ABCG2^+/CD24^-$	Explanted Primary Human Prostate Tumour	High clonogenic potential; Prostatosphere formation; Immunohistochemistry for CD133, CD44 and ABCG2 in prostate cancer biopsies.	(25)
$PSA^{-/low}/ALDH^+/CD44^+/\ \alpha_2\beta_1^+$	Prostate Cancer Cell Lines, Prostate Cancer Xenografts	Tumour formation in mice at low titres. Increased cell frequency following castration.	(18)

Figure Captions

Figure 1. Summary of the covered methods and markers used to characterise prostate cancer stem cells.

Table 1. Examples of expression profiles recently found to identify putative human prostate CSCs.